REMARKS

Favorable reconsideration is respectfully request in view of the foregoing amendments and following remarks.

Claim 2 was canceled in the Applicants' last response.

Claim 1 has been amended to incorporate the subject matter of claim 10, and claim 10 has accordingly been canceled.

Claim 1 has further been amended to specify a lower limit of the range of thickness of the tissue section, so as to now recite a thickness of tissue section from 0.5 to 50 μm. Support is found in the specification at page 21, line 6. Claim 1 has further been amended to specify that the tissue section-containing carrier is for seeding and culturing animal cells. Support is found in the Examples. In addition, claim 1 has been amended to specify that the tissue section is pre-balanced with a culture growth medium, as taught in the Examples.

Claims 1, 3-6, 8-9 and 11-23 are pending after the foregoing amendments.

Turning to the Official Action, claims 1-6 and 8-13 were rejected under 35 U.S.C. 112, first paragraph, on the basis that the phrase "not to exceed" fails to comply with the written description requirement.

The phrase objected to by the Examiner has been removed and a specific lower limit has been introduced into claim 1.

Accordingly, this ground of rejection is deemed to be overcome.

Claims 1-6, 8-11 and 13 were rejected under 35 U.S.C. 102 as anticipated by U.S. 5,919,624.

Claim 1 has been amended to specify that the tissue section is pre-balanced with a culture medium.

The cited reference fails to disclose a tissue section which is pre-balanced with a culture medium. All of the tissue sections described in the '624 patent are not in contact with a culture medium.

Accordingly, this ground of rejection is deemed to be overcome.

Lastly, claims 1-6 and 8-13 were rejected under 35 U.S.C. 103 as obvious over Mori et al. and/or WO 99/12555 taken with the '624 patent and U.S. 3,785,234. This ground of rejection is respectfully traversed.

It is respectfully submitted that the combined teachings of the cited references would not motivate one of ordinary skill in the art to prepare an animal-derived or plant-derived tissue section-containing carrier according to the claimed invention.

Mori et al. describe cutting neonatal mouse liver tissues into 250 μm slices and culturing the tissue to maintain the parenchymal cells in ontogenesis and to investigate their proliferation and differentiation.

Mori et al. fail to disclose or suggest the use of an animal-derived or plant-derived tissue section in a thickness of 0.5 to 50 μm used as a substrate for seeding and culturing animal cells.

WO 99/12555 is generally directed to the use of submucosal tissue which is enzymatically treated with galactosidase which can be implanted to replace or support damaged or diseased tissues or to form a cell culture growth substrate. The submucosal tissue has a thickness of about 100 to 200 μm and consists primarily of acellular, extracellular matrix material. See the Abstract and page 4, lines 30-32.

There is no suggestion in the reference that other tissues sections from animals, or plants, may be used as a cell culture growth substrate, nor that the cell culture substrate may have a thickness of from 0.5 to 50 μm.

The '624 patent is directed to methods of detecting the presence of cervical carcinoma by examining tissue sections. There is no teaching or suggestion of using the tissue section as a cell culture growth substrate.

Lastly, the '234 patent is directed to devices for cutting thin tissue sections.

Nowhere in the combined teachings of the cited references is there a suggestion of the claimed invention, which is a tissue section-containing carrier derived from animal or plant, for seeding and culturing animal cells, wherein the tissue section is stuck or stuck while stretched to a substrate and is pre-balanced with a culture medium, and furthermore wherein the tissue section has a thickness of from 0.5 to 50 μm.

For the Examiner's information, there is submitted herewith a publication by the Inventors in the FASEB Journal, Volume 16, pages 1847-1849, November 2002, describing the instant invention.

Favorable reconsideration and allowance is respectfully solicited.

Respectfully submitted,

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Cell culture on thin tissue sections commonly prepared for histopathology[1]

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SPECIFIC AIMS

The aim of our study was to establish a breakthrough technology to study cellomics based on cell–substratum interactions. We established a novel culture method of animal cells using a substratum made of tissue/organ sections for histopathology (TOSHI substratum) and investigated its unique advantages using four types of cells: BeWo (human choriocarcinoma cell line) cells, CPAE (bovine pulmonary artery endothelial cell line) cells, PC-12 (rat pheochromocytoma cell line) cells, and NHDFs (normal human dermal fibroblasts).

PRINCIPAL FINDINGS

1. Preparation of TOSHI substrata made of a bovine placenta

To investigate the behavior of cells cultured on substrata made of thin tissue sections, two types of substrata from bovine placenta cryosections were prepared in a culture tray: section substratum and acellularized section substratum. One type of substratum contained cellular components and the other did not. Frozen sections from a bovine placenta embedded in an OCT compound were spread on glass slides and air-dried. Each section included the three regions of the placenta, the labyrinth of the cotyledon, the basal lamina of the endometrium, and the glandular zone of the endometrium. To prepare the general type of TOSHI substratum, each section-mounted glass slide was placed in a culture tray and immersed consecutively in Hank's balanced salt solution (HBSS) for 5 min to remove the OCT compound, in 70% ethanol for 10 min to fix and sterilize the section, in HBSS for 5 min to completely remove the ethanol, and in a basal medium for 5 min to optimize the section as a culture substratum. To prepare the acellular type of TOSHI substratum, two additional steps were introduced before the 70% ethanol treatment: the section-mounted glass slide was immersed in 0.1% sodium dodecyl sulfate (SDS) in HBSS for 20 min to dissolve cellular components, followed by HBSS for 5 min to remove the SDS and cell lysates.

2. Morphogenesis of BeWo cells and CPAE cells cultured on the TOSHI substrata prepared from a bovine placenta

BeWo cells were seeded and cultured on the section substrata of varying thickness (5, 10, or 20 μm) and cell morphology was compared. Cells cultured on the basal lamina and glandular zone of the section substratum as well as on the glass slide substratum formed flat colonies consisting of cobblestone-shaped cells. As the section thickness increased, cells on the labyrinth portion of the section substratum tended to form aggregates of cuboidal cells. On culture day 3, cells on the labyrinth portion of the 20 μm section substratum formed multicellular spheroids of 50–100 μm in diameter. This demonstrated that the labyrinth region of the substratum had the potential to facilitate BeWo cell self-assembly.

CPAE cells were seeded and cultured on the acellularized section substratum to investigate the behavior of endothelial cells. Cells attached and spread well over all regions of the substratum after 1 day of culture. Through culture day 3, cells grown on the labyrinth region formed a capillary network-like structure involving the microarchitecture of the acellularized section, but cells grown on the basal lamina and glandular zones merely proliferated up to a confluent monolayer of cobblestone-shaped cells. Using laser scanning confocal microscopy, 3-dimensional morphology of the capillary-like structure showed that intertwining CPAE cells reconstructed a 3-dimensional tube of ~5 μm in diameter. This result demonstrated that the labyrinth region of the substratum could induce differentiation in CPAE cells.

3. Serum-free culture of PC-12 cells on TOSHI substrata prepared from a bovine placenta

To examine changes in cell viability and behavior on serum depletion, PC-12 cells were suspended in a

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02-0405fje; to cite this article, use *FASEB J.* (September 5, 2002) 10.1096/fj.02-0405fje

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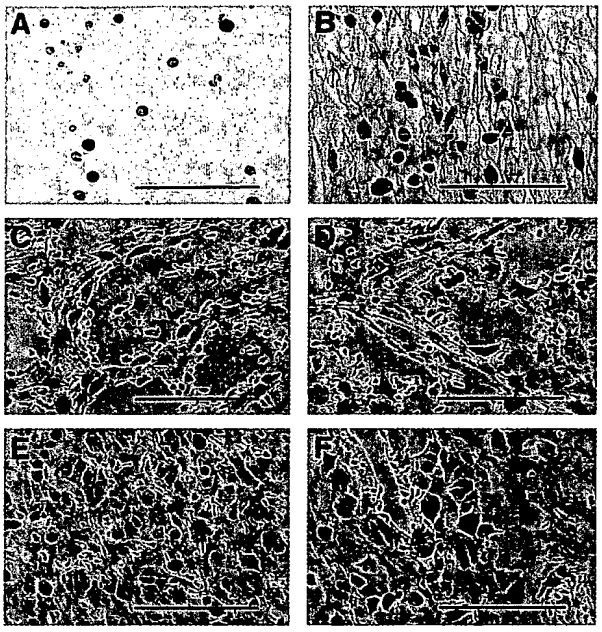

Figure 1. Serum-free culture of PC-12 cells on placenta cryosection substrata. Cells were suspended in a serum-free medium and seeded at a density of $4.6 \times 10^4/cm^2$ and cultured on a glass slide substratum as well as on 5 μm section or 5 μm acellularized section substrata. After HE staining, the morphology of cells cultured on the glass slide (*A*), acellularized basal lamina (*B*), labyrinth (*C, D*), basal lamina (*E*), and glandular zone (*F*) for 2 days (*A–C*) or 5 days (*D–F*) was observed by optical microscopy. Scale bars, 100 μm.

serum-free medium, seeded, and cultured either directly on a glass slide substratum or on the section substratum or acellularized section substratum. The few cells that adhered to the glass slide substratum did not spread and exhibited an apoptotic cell morphology with plasma membrane blebbing at culture day 2. In contrast, most cells seeded on the acellularized section substratum spread well and exhibited a healthy cell morphology with cobblestone shape on its all regions, demonstrating that this substratum has the potential to prohibit the onset of apoptosis by facilitating cell adhesion and spreading even under serum-free conditions. The cells seeded on the section substratum spread well on all regions at day 2, after which some cells particularly on the labyrinth region differentiated and formed a neuronal network-like structure through day 5. Cells on the basal lamina region and glandular zone maintained their healthy cell morphology and cobblestone shape (**Fig. 1**). These results demonstrated that the two types of substrata provided a microenvironment that maintained cell viability in the absence of serum and that the labyrinth region of the section substratum has the potential to induce differentiation in PC-12 cells.

4. Formation of a 3-dimensional multicellular aggregate of NHDFs involving the acellularized section-derived ECM components

NHDFs were seeded and cultured on the acellularized section substratum to investigate the behavior of mesenchymal cells. The fibroblasts proliferated and formed multilayers on all regions of the substratum. The multilayered fibroblasts comprising the substratum began to spontaneously detach from the glass slide at culture day 7 because adherence of the acellularized section substratum was altered by proliferating fibroblasts. Self-supporting sheet consisting of the acellularized section and fibroblast layers could be completely detached from the glass slide by gently pipetting the culture medium to produce shear stress. The detached fibroblast sheet was cultured for 2 additional days on a nonadhesive substratum, resulting in a 3-dimensional multicellular aggregate incorporated the acellularized section-derived ECM components. The aggregate was ~3.0 mm along the long axis and ~1.5 mm along the short axis. The interior histology of the aggregate was different in the outer side and the inner side. The outer side contained the majority of fibroblasts that were self-assembled and incorporated only a small amount of the acellularized section substratum-derived compo-

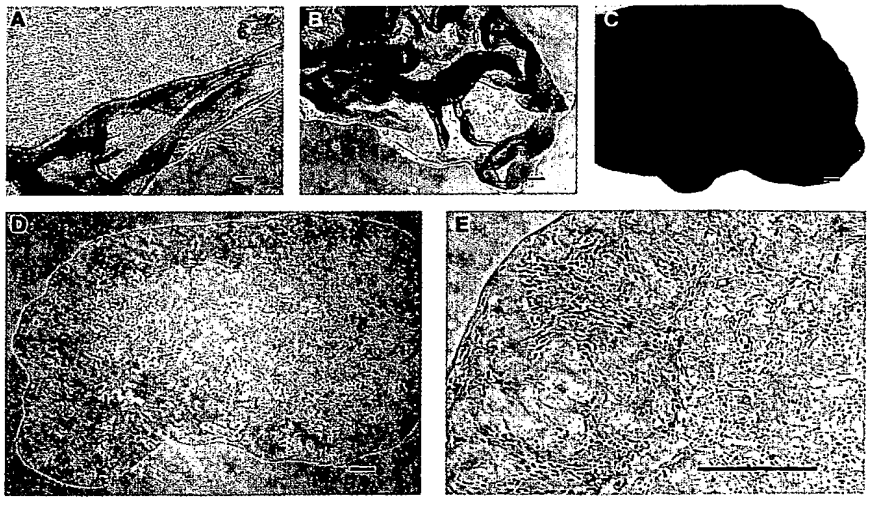

Figure 2. Formation of a 3-dimensional multicellular aggregate of normal human dermal fibroblasts containing the acellularized placenta cryosection substratum-derived components. Cells were seeded at a density of $3.3 \times 10^4/cm^2$ and cultured on a 20 μm acellularized section substratum. Proliferating cells cultured on the acellularized section substratum for 7 days began to detach from the glass slide (*A*) and a cell sheet containing the acellularized section substratum could be completely detached by pipetting the culture medium (*B*). A 3-dimensional multicellular aggregate containing the acellularized section substratum-derived components was formed after an additional 2 days of culture on a nonadhesive substratum (*C*). The morphology of cells in culture was observed by phase-contrast microscopy (*A–C*). HE-stained cross sections of the aggregate were observed by optical microscopy (*D, E*). Scale bars, 200 μm.

nents in a twisted shape. The inner side contained a minority of fibroblasts associated with a large amount of the acellularized section substratum-derived components in a twisted shape. The edge of the aggregate was covered with squamous fibroblasts (**Fig. 2**). This culture process revealed that a mass of mesenchymal cells could be prepared using the acellular type of TOSHI substratum and that ECM components of the acellular substratum were incorporated into the multicellular mass.

CONCLUSIONS AND SIGNIFICANCE

We established a breakthrough technology based on the concept of culturing animal cells on a substratum made of tissue/organ sections for histopathology (TOSHI-substratum). We demonstrated the application of TOSHI substrata prepared from a bovine placenta in tissue reconstruction and a serum-free culture. Our data clearly show that differentiation of BeWo, CPAE, and PC-12 cells is induced only on the labyrinth region of the substratum, that the substratum provides a microenvironment sufficient to maintain the viability of PC-12 cells under serum-free conditions, and that a multicellular mass of NHDFs involving acellularized section-derived components can be prepared by using the substratum. The multicellular mass incorporating the section components derived from the TOSHI substratum may have important clinical applications as an implant since it is easily prepared using cryosections and cells derived from patient biopsies. The detailed molecular mechanisms underlying each phenomenon can be investigated using monoclonal antibodies and/or digestive enzymes directed against molecules expressed in the bovine placenta.

Our novel procedure of culturing cells on TOSHI substrata has several potential applications for basic life science and applied biomedical research. TOSHI substrata can be prepared not only from all animal tissues/organs of any age regardless of pathology, but also from

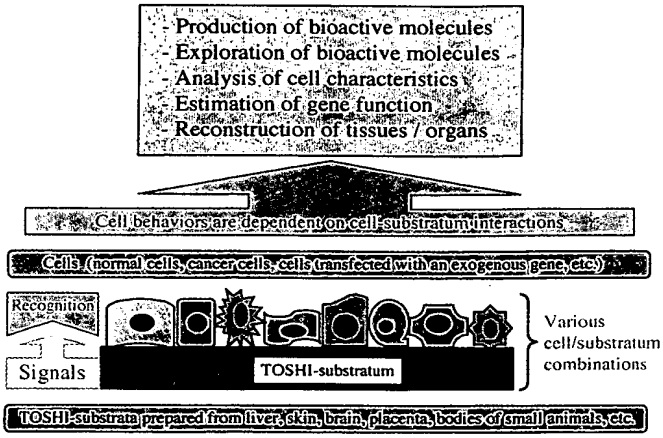

Figure 3. The potential of the novel TOSHI substratum for cellomics studies. The study of cell behavior depends on cell–substratum interactions. Potential applications include research to enhance the secretion levels of specific bioactive molecules by selecting an appropriate TOSHI substratum, explore key molecules within TOSHI substrata that regulate cell behavior, extrapolate cell properties (e.g., cancer cells) via cell affinity for a specific region on a TOSHI substratum (e.g., whole-body section substratum), estimate gene function by measuring differences in cell behavior on TOSHI substrata before and after transfection of an exogenous gene, and reconstruct organoids in which the differentiation of each cell is regulated by exploiting the TOSHI substrata that conserve hierarchical cellular architecture (cell lineage).

the entire body of small animals. These substrata retain the original microarchitecture and composition of the tissue and conserve many biochemical factors that serve as signaling cues for inducing cell behavior. Most of these factors are easily detected by ordinary techniques such as immunohistochemistry or in situ hybridization. This work sets the stage for the analysis of interactions between different cell types and various TOSHI substrata, and offers a novel approach for studies in cellomics. In the near future, delineating the proper cell–substratum combination will promote effective cellomics studies (**Fig. 3**). FJ